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Glutamatergic Inhibition of Voltage-Operated Calcium Channels in the Avian Cochlear Nucleus

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The auditory nerve serves as the only excitatory input to neurons in the avian cochlear nucleus, nucleus magnocellularis (NM). NM neurons in immature animals are dependent upon auditory nerve signals; when deprived of them, many NM neurons die, and the rest atrophy. Auditory nerve terminals release glutamate, which can stimulate second messenger systems by activating a metabotropic glutamate receptor (mGluR). Therefore, it is possible that the effectors of mGluR-stimulated signal transduction systems are needed for NM neuronal survival. This study shows that mGluR activation in NM neurons attenuates voltage-dependent changes in $[Ca^{2+}]_i$. Voltage-dependent Ca^{2+} influx was also attenuated by increasing cAMP with forskolin, VIP, or 8-bromo-cAMP, indicating that mGluR activation may stimulate adenylate cyclase. The main results may be summarized as follows. NM neurons possess high voltage-activated Ca^{2+} channels that were modulated by quisqualate, glutamate, and (±)*trans*-ACPD, in that order of potency. Glutamatergic inhibition of Ca^{2+} influx was not blocked by L-AP3 or L-AP4, which antagonize the actions of mGluRs in other neural systems; it was blocked by serine-*O*-phosphate. Finally, the attenuation of voltage-dependent Ca^{2+} influx was duplicated by cAMP accumulators. Since NM neurons have high rates of spontaneous activity and higher rates of driven activity, the expression of this mGluR turns out to be very valuable: without it, $[Ca^{2+}]_i$ could reach lethal concentrations. These results provide an important clue as to the identity of an intracellular signal that may play an important role in NM neuronal survival.

[Key words: deafferentation, excitatory amino acid receptors, neuroprotection, cAMP, auditory system, metabotropic receptor]

In mammals and birds, removal of the membranous cochlea abolishes auditory nerve activity. This initiates a cascade of events which, in young animals, culminates in transneuronal death and atrophy of neurons in the cochlear nucleus. The cellular events underlying these changes have been most thoroughly studied in nucleus magnocellularis (NM) of the neonatal chicken (see Rubel et al., 1990, for review). Accordingly, it has been postulated that auditory nerve axons provide NM neurons with signals required for neural information processing, and signals that facilitate, promote, or are essential to neuronal survival. Physical contact of the eighth nerve terminal on NM neurons does not promote neuronal survival since degenerative changes are seen when action potentials of the auditory nerve are eliminated by intralabyrinthine application of the sodium channel blocker tetrodotoxin (Born and Rubel, 1988; Pasic and Rubel, 1989). These results indicate that an intracellular process associated with the excitation of NM neurons is required. However, activity per se is not sufficient: antidromic stimulation of NM neurons or stimulation of eighth nerve axons in a Ca^{2+}/Mg^{2+}-free environment (eliminating postsynaptic activation of NM neurons) does not prevent degenerative changes from occurring (Hyson and Rubel, 1989). Degenerative changes are not seen in orthodromically stimulated neurons, however, indicating that this activity-dependent trophic process is dependent upon a presynaptically released agent. The excitatory amino acid (EAA), glutamate, is believed to be released from auditory nerve terminals, acting as an excitatory transmitter at the eighth nerve-NM neuron synapse (Nemeth et al., 1983; Raman and Trussell, 1992; Zhou and Parks, 1992). In the present study, we show that glutamate stimulates a second-messenger system in NM neurons whose effectors prevent lethal concentrations of Ca^{2+} from accumulating.

In addition to stimulating ionotropic receptors (iGluRs) involved in generating EPSPs, glutamate activates metabotropic glutamate receptors (mGluR) that stimulate signal transduction pathways. As a result, mGluRs are capable of altering cell physiology (Desai and Conn, 1991; Lovinger, 1991; Glaum et al., 1992; Collins, 1993), ion channels (Bleakman et al., 1992; Glaum and Miller, 1992, 1993; Kelso et al., 1992; Kinney and Slater, 1993; Sahara and Westbrook, 1993), and neuronal responses to potentially toxic events (Chiamulera et al., 1992; Optiz and Reymann, 1993; Pizzi et al., 1993). In the course of examining the role of glutamate release and its actions on intracellular Ca^{2+} responses of NM neurons (Zirpel et al., 1994b), it was discovered that glutamate activates a mGluR that inhibits the influx of Ca^{2+} in depolarized neurons. The EAA agonists kainate and AMPA do not mimic this effect; however, quisqualate, a nonspecific mGluR agonist, and ±*trans*-ACPD (*t*-ACPD), a specific mGluR agonist, do impede Ca^{2+} influx. This study describes this effect and one of the potential intracellular signals that may regulate it. The results are consistent with the

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hypothesis that activation of this mGluR in NM neurons prevents toxic concentrations of Ca^{2+} from accumulating. Thus, the products of mGluR stimulation may be essential to the survival of NM neurons, and the absence of these products may initiate or contribute to the degenerative changes observed following deafferentation.

Materials and Methods

Tissue preparation. White leghorn chicken embryos, 17–18 d old (E17–E18), were removed from their shells and staged according to the criteria of Hamburger and Hamilton (1951). The embryo was decapitated and its cerebellum was removed via gross transection of the cerebellar peduncles. The exposed brainstem was blocked rostrally at the level of the motor nucleus of the trigeminal nerve and caudally at the level of the glossopharyngeal nucleus. This block was removed and immersed in 5% low melting point agarose dissolved in Geys buffer. A square block containing the brainstem was cut out after the agarose solidified, glued onto a metal stage, and cut with a vibratome. A total of 3 min typically elapsed between the time the embryo was decapitated and the first vibratome section was made.

Coronal sections, 300 μm thick, were cut into cold, oxygenated artificial cerebrospinal fluid (aCSF). The aCSF was composed of 125 mM NaCl, 5 mM KCl, 1.25 mM KH_2PO_4, 1.3 mM $MgCl_2$, 26 mM sodium bicarbonate, 10 mM dextrose, and 3.1 mM $CaCl_2$. A pair of sections containing the rostral one-half of NM were dissected free of agarose, choroid plexus, and meninges and transferred to an aCSF solution containing 5 μM fura-2 acetoxymethyl ester (Fura-2), 0.1% dimethylsulfoxide, and 0.02% pluronic acid. The slices remained in this solution, oxygenated, and warmed to 37°C for 20 to 35 min, at which point they were rinsed in oxygenated aCSF for 10 min as they were being prepared for imaging. Only one slice was examined per animal, and this slice was not exposed to more than one type of glutamate agonist. This strategy was adopted to prevent confounding results caused by the activation of a second-messenger system stimulated by one agonist that might alter the Ca^{2+} responses of a second agonist. A single experiment was replicated at least three times, and a single slice from a single animal was used for each experiment.

Microfluorometry. Fura-2 loaded NM cells were alternately illuminated with 340 nm and 380 nm wavelengths from a xenon source (Osram). Excitation wavelengths were obtained using interference filters from Chroma Technology (Brattleboro, VT). Emitted light was passed through a 40× fluor oil immersion objective (Nikon) attached to a Nikon Diaphot inverted microscope, through a 480 nm long-pass exit filter, and finally into an image intensifier coupled to a CCD camera (Hamamatsu, Japan). Cells were exposed to UV light, attenuated to 3% its normal intensity by neutral density filters, during data collection periods only. Exposure time for each wavelength was between 500 to 750 msec, using a computer-controlled shutter and filter wheel (Sutter Inst., Novato, CA). Paired images were captured every 3 sec. Data were obtained by comparing the intensity of fluorescent emission to 340 nm and 380 nm excitation wavelengths. The difference in emitted fluorescence was expressed as a ratio (F340:F380) that was compared to a standard curve for free Ca^{2+} constructed from solutions of known Ca^{2+} and fura-2 concentrations. As a result, ratios of fluorescent intensity were translated directly to Ca^{2+} concentrations using software designed by Universal Imaging Corp. (West Chester, PA). The K_d of hydrolyzed fura-2 for Ca^{2+} was calculated as 224 nM (Grynkiewicz et al., 1985). Numerical values reported are an average of > 50 adjacent pixels.

Pharmaceuticals. The Results section is composed of three components. The first describes the Ca^{2+} response evoked by depolarizations using 60 mM KCl, and shows that NM neurons possess L-type Ca^{2+} channels, but not N-type channels. The dihydropyridines, BAY K 8644, and nifedipine were used to test for the presence of L-channels, and omega-conotoxin GVIA (ω-CgTX) was used to test for N-channels.

The next segment of the Results describes the effects that glutamate and its analogs have on voltage-dependent Ca^{2+} influx. The glutamate analogs kainate and alpha-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA) were tested, as were the nonspecific and specific mGluR-agonists quisqualate (QA) and ± *trans* 1-amino-1,3 cyclopentanedicarboxylic acid (*t*-ACPD), respectively. The actions of glutamate on voltage-dependent Ca^{2+} influx were challenged by 6 cyano-7-dinitroquinoxaline-2,3 dione (CNQX), an antagonist of non-NMDA-type glutamate receptors, as well as L(+)-2-amino-3-phosphonopropionate (L-AP3), (±)-2-amino-4-phosphonobutyric acid (L-AP4), and L-serine-*O*-phosphate (SOP), which have all been shown to block the actions of mGluRs in other neural systems.

The final segment describes the effects that accumulators of cAMP had on voltage-dependent Ca^{2+} influx. Specifically, NM neurons were exposed to forskolin and vasoactive intestinal peptide (VIP), accumulators of adenylate cyclase; a membrane permeable analog of cAMP, 8-bromo-adenosine cyclic 3′,5′-hydrogen phosphate monosodium salt (8-Br-cAMP); and a pair of phosphodiesterase inhibitors, 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro 20-1724), and 3-isobutyl-1-methylxanthine (IBMX). Neurons were also exposed to a protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA).

With a few exceptions, drugs were first dissolved as 200 to 1000× stock solutions and frozen at −40°C or at −70°C until use. Stock solutions were all dissolved in water, with the following exceptions: BAY-K 8644 was dissolved in methanol; L-AP4, forskolin, IBMX and Ro 20-1724 were dissolved in ethanol; nifedipine, CNQX, and PMA were dissolved in dimethylsulfoxide. Working solutions of *t*-ACPD, L-AP3, L-AP4, and SOP were freshly prepared for each experiment. Solutions of aCSF and glutamate were also prepared daily.

Drug application. A brainstem slice containing NM was placed in Leiden-type microscope-stage chamber, and anchored to the floor of the chamber with a custom-made stainless steel net. A 1 mm thick, 25 mm diameter coverglass served as the floor of the chamber, creating a 10 mm deep reservoir. A stainless steel capillary inlet tube (1 mm i.d.) was located 1 mm above the slice, while the capillary tube connected to a vacuum pump rested on the reservoir floor. The slice was continually perfused in aCSF. The fluid volume in the reservoir when it contained a slice was estimated to be 250 μl, and the laminar flow rate of the gravity-delivered perfusion system was 3 ml/min. The tubing connecting the 60 cc media reservoirs to the recording chamber was approximately 30 cm long. As a result, a complete change in the composition of the aCSF from a control solution to a test solution was delayed by approximately 15 to 20 sec. The figures presented in the Results section have not been corrected for this delay. Overall, this system permitted a rapid and homogeneous application, and equally rapid elimination of drugs. All drugs were dissolved and delivered in the perfusate (i.e., aCSF). Solutions used to depolarize NM neurons contained 60 mM KCl. Calcium-free solutions were obtained by adding 5 μM EGTA to nominally Ca^{2+}-free aCSF. When testing for the effects of drugs under Ca^{2+}-free conditions, slices were superfused with Ca^{2+}-free aCSF for at least 2 min before and after agonist application.

Materials. Fura-2 was obtained from Molecular Probes, Inc. (Eugene, OR). Pharmaceuticals, with the exception of ω-CgTX, VIP, and glutamate, were purchased from Research Biochemicals, Inc. (Natick, MA). ω-CgTX was purchased from Peninsula Laboratories (Belmont, CA), while VIP was purchased from Calbiochem (La Jolla, CA). Glutamate was acquired from Sigma. All other reagents were of analytical grade.

Results

Figure 1*A* shows a photomicrograph of the avian nucleus magnocellularis (NM). The nucleus is composed of a single cell type, shown here stained with antibodies against microtubule-associated protein 2 which is used to reveal dendritic arborizations. These spherically shaped cells are roughly 30 μm in diameter, and adendritic at this age (Boord, 1969; Parks and Rubel, 1978; Smith and Rubel, 1979; Jhaveri and Morest, 1982). A single neuron is usually innervated by two to three auditory nerve axons that terminate with endbulbs of Held. Figure 1*B* shows a micrograph of NM neurons filled with Fura-2. Although there was some variation in the amount of indicator taken up by neurons, the cytosol was uniformly fluorescent, and the fluorescent emission was intense. In some neurons, the fluorescent intensity of the nucleus was noticeably greater than the fluorescent intensity of the cytoplasm.

The average basal intracellular calcium ion concentration of the neurons examined in this study was 78 ± 25 nM (SEM). A few neurons exhibited high basal $[Ca^{2+}]_i$, some in the neighborhood of 200 nM, others exceeding 1000 nM. These cells were presumably damaged by the dissection and were not evaluated. Of the 25 to 30 cells visible in the microscopic field, approxi-

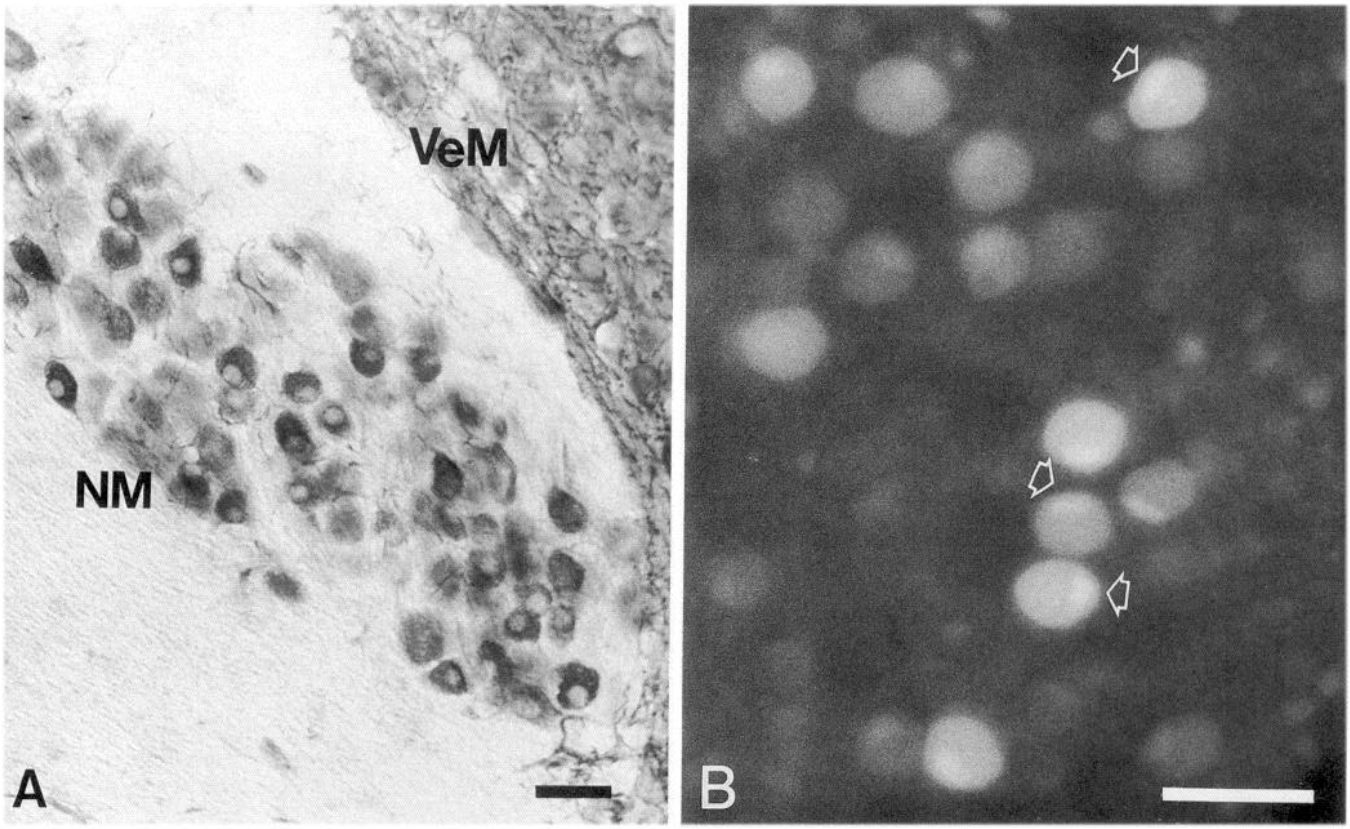

Figure 1. Nucleus magnocellularis (NM) neurons stained for microtubule-associated protein 2 (MAP-2) and Fura-2. *A,* As revealed by MAP-2 staining, NM is composed of round, adendritic neurons. *B,* This micrograph shows FURA-2–labeled neurons. In some neurons, the fluorescent intensity of the nucleus was significantly greater than that of the cytosol (*arrows*). *VeM*, medial vestibular nucleus; *NM*, nucleus magnocellularis. Scale bars, 40 μm.

mately 10% were excluded from analyses because of elevated $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ are reported as the mean ± SEM. The total number of neurons examined (attended parenthetically by the number of slices used) is reported below.

NM neurons possess at least two different VOCCs

The graph in Figure 2*A* plots, over time, the change in $[Ca^{2+}]_i$ (Ca^{2+} response) that was seen in a single neuron depolarized by 60 mM KCl. The onset of KCl stimulation is indicated by the left-most dotted line, while the termination of KCl stimulation is shown by the second vertical line. Superfusion of 60 mM KCl caused an increase in $[Ca^{2+}]_i$. The kinetic and temporal properties of the Ca^{2+} response in 64 depolarized neurons ($n = 7$) examined were highly stereotypic: a slow, but brief increase in $[Ca^{2+}]_i$ was initially seen followed by a sudden, rapid, increase to 977 ± 193 nM (a Ca^{2+} transient). An equally rapid decay to near basal levels occurred after the $[K^+]_o$ was returned to normal levels. The complete return to basal $[Ca^{2+}]_i$ was protracted, however; 91% of the neurons reestablished basal $[Ca^{2+}]_i$ 5 to 7 min after the depolarization. Figure 2*B* shows the Ca^{2+} responses of eight neurons simultaneously imaged. The Ca^{2+} response caused by elevating $[K+]_o$ could be evoked repeatedly, and the magnitude of the response and its temporal properties did not vary significantly between depolarizations. In the experiment illustrated in Figure 2*B*, these neurons were depolarized six times, each time by a 20 sec-long superfusion of 60 mM KCl. These results show that the simple influx of Ca^{2+} (or the mere exposure to KCl) does not affect the magnitude of subsequent Ca^{2+} responses produced by KCl. Ca^{2+} transients were not observed when NM neurons were depolarized in Ca^{2+}-free aCSF.

The influx of Ca^{2+} in NM neurons can be carried by a single or a set of voltage-operated Ca^{2+} channels (VOCCs); three different channels have been identified: L-, N-, and T-type (Nowycky et al., 1985; Fox et al., 1987a,b; see Bean, 1989, for review). The L- and N-type channels are activated by large changes in membrane voltage, while the T-type channels are activated by smaller changes in membrane voltage. Since it is most likely that the Ca^{2+} transients produced by 60 mM KCl are carried by high voltage activated (HVA) L- or N-type channels, rather than low voltage activated (LVA), or T-type channels, the effects of HVA agonists and antagonists were tested on KCl-stimulated Ca^{2+} influx. Specifically, dihydropyridine (DHP) agonists and antagonists were used to determine if NM neurons express L-channels (see Carbone and Swandulla, 1989; Sher et al., 1991, for review of DHP actions); ω-CgTX was used to test for N-channels (see Fox et al., 1987a).

NM neurons express L-type channels. The effect of BAY K 8644 was examined on 24 neurons ($n = 3$ slices) depolarized by 30 mM KCl. As seen in Figure 3*A*, the change in $[Ca^{2+}]_i$ caused by elevating the $[K+]_o$ to 30 mM was negligible (inset, Fig. 3*A*). A larger influx of Ca^{2+} was seen when 30 mM KCl and 10 μM BAY K 8644 were coapplied. In the presence of 30 mM KCl alone, $[Ca^{2+}]_i$ increased to 87 ± 22 nM. The $[Ca^{2+}]_i$ increased to 161 ± 33 nM when KCl and BAY K 8644 were coapplied.

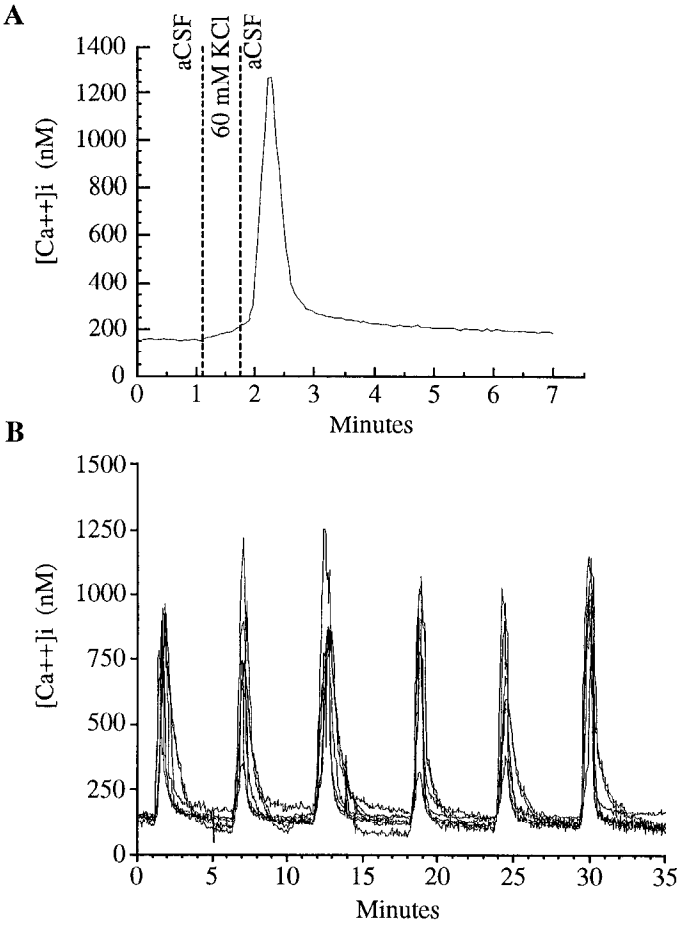

Figure 2. Voltage-dependent Ca^{2+} response of NM neurons. *A,* When depolarized by 60 mM KCl, neurons responded with a large increase in $[Ca^{2+}]_i$. A calcium transient for a single NM neuron is illustrated here. *B,* Calcium responses for eight neurons are plotted, depolarized by six sequential applications of KCl. Large Ca^{2+} transients were observed each time 60 mM KCl was superfused.

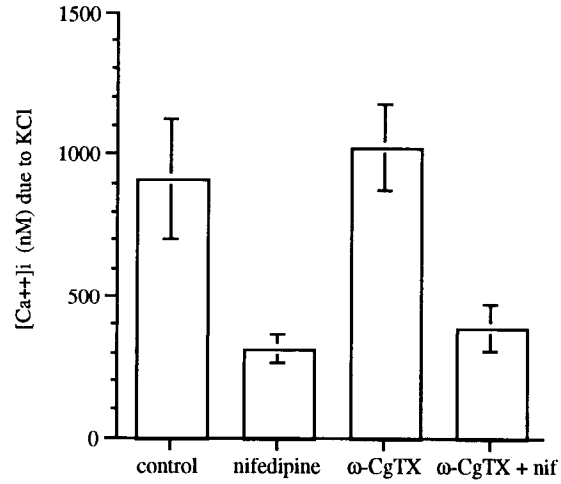

Figure 4. NM neurons do not possess ω-CgTX-conotoxin sensitive Ca^{2+} channels. *A,* These histograms show the $[Ca^{2+}]_i$ seen in NM neurons depolarized by 60 mM KCl under four different conditions: control (normal aCSF), in the presence of 1 μM nifedipine, following a 15 min preincubation in 2.5 μM ω-CgTX conotoxin, and in the presence of 1 μM nifedipine following ω-CgTX incubation. A Student's *t* test revealed that nifedipine significantly attenuated the increase in $[Ca^{2+}]_i$ due to KCl ($p < 0.001$). The change in $[Ca^{2+}]_i$ seen in KCl-depolarized neurons preincubated in ω-CgTX did not differ statistically from controls.

Whereas the DHP agonist BAY K 8644 augmented Ca^{2+} influx, the DHP antagonist nifedipine attenuated Ca^{2+} influx. A total of 41 ($n = 5$) neurons were examined. The coapplication of 500 nM nifedipine and 60 mM KCl did not completely block Ca^{2+} influx (Fig. 3*B*): small elevations in $[Ca^{2+}]_i$ were seen, but large Ca^{2+} transients were not. In the absence of nifedipine, the mean $[Ca^{2+}]_i$ increased to 912 ± 213 nM; in the presence of nifedipine, the mean $[Ca^{2+}]_i$ increased to 310 ± 53 nM. The Ca^{2+} responses of NM neurons preincubated in 2.5 μM ω-CgTX (see Fig. 4) and depolarized in the absence and presence of 500 nM nifedipine did not differ significantly from the responses of

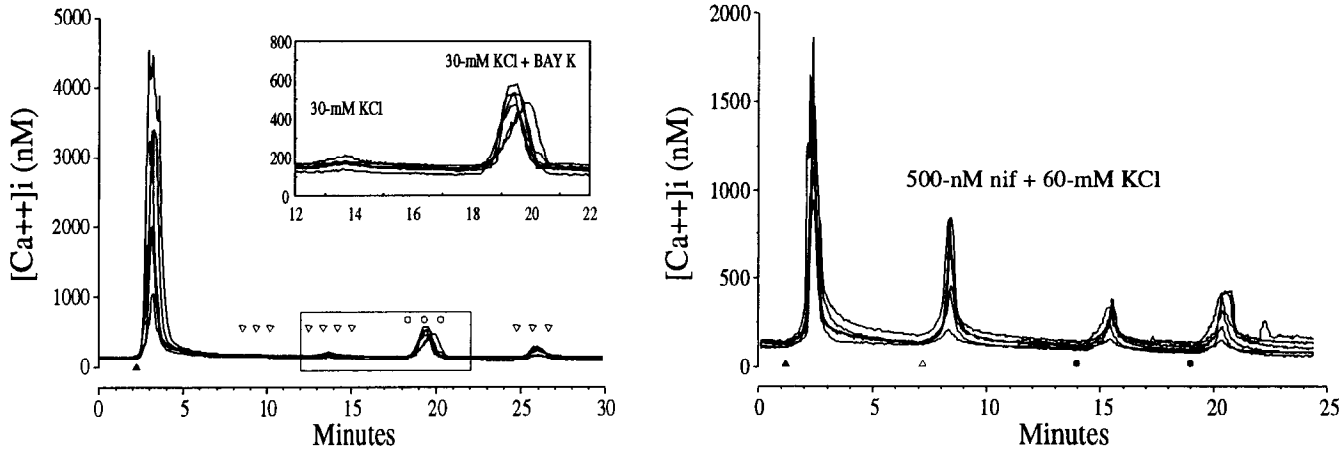

Figure 3. Dihydropyridines (DHP) alter the Ca^{2+} responses of NM neurons depolarized by elevated $[K+]_o$. *A,* Calcium responses of neurons depolarized by 60 mM KCl for 20 sec, by 30 mM KCl for 2 min, and by 30 mM KCl + 10 μM BAY K 8644 for 2 min, are shown for six neurons. KCl (60 mM) (*black triangle*) caused the $[Ca^{2+}]_i$ to significantly rise. A smaller change in $[Ca^{2+}]_i$ was produced by 30 mM KCl (*white triangles*, and see *inset*). A larger change in $[Ca^{2+}]_i$ was seen when 30 mM KCl and the DHP agonist BAY K 8644 (*white circles*) were coapplied. *B,* Calcium responses of five neurons depolarized by 60 mM KCl before (*black triangle*), during (*white triangle*), and after (*black circles*) neurons were exposed to the DHP "antagonist" nifedipine (500 nM). Following nifedipine exposure, large Ca^{2+} transients were no longer observed.

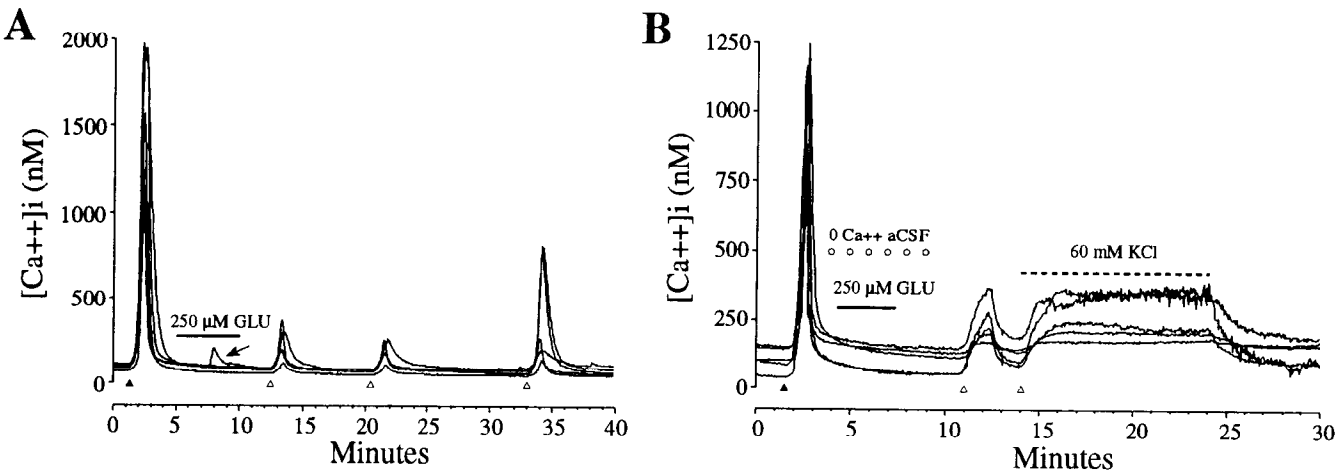

Figure 5. Glutamate attenuates voltage-dependent Ca^{2+} influx. *A*, The Ca^{2+} responses of five neurons to 20 sec long pulses of 60 mM KCl before (*black triangle*) and after (*white triangles*) superfusion of 250 μM glutamate (*GLU*) are plotted. The *black bar* marks the duration of glutamate superfusion. Preceding GLU exposure, all neurons responded to 60 mM KCl with stereotypic Ca^{2+} transients. During GLU superfusion, only one neuron (*arrow*) showed an increase in $[Ca^{2+}]_i$. Following GLU exposure, neurons depolarized by KCl showed markedly attenuated Ca^{2+} responses. *B*, Shown here are Ca^{2+} responses of five neurons depolarized by 60 mM KCl before and after superfusion of 250 μM glutamate applied in Ca^{2+}-free (0 Ca^{2+}) aCSF. The duration of the 0 Ca^{2+} aCSF is markedly by *white circles*. The first transient (*black triangle*) was produced by a 20 sec-long pulse of 60 mM KCl . Not one neuron responded with an increase in $[Ca^{2+}]_i$ during the superfusion of 0 Ca^{2+} GLU (duration is indicated with the *black bar*). Following GLU exposure, neurons were rinsed for 3 min in normal aCSF (*white triangles*), then depolarized with 60 mM KCl, first for 2 min, then for 10 min (indicated by the *dashed bar*).

neurons that were not exposed to ω-CgTX ($[Ca^{2+}]_i$ for ω-CgTX calone = 1023 ± 151 nM vs. ω-CgTX + nifedipine = 388 ± 84 nM).

In summary, NM neurons responded in a predictable fashion when they were depolarized by elevated $[K+]_o$: a rapidly developing large Ca^{2+} transient was produced that could be blocked with the L-channel antagonist, nifedipine. Ca^{2+} influx in depolarized NM neurons was not changed by ω-CgTX, indicating that NM neurons at this age do not possess N-type calcium channels.

Glutamate attenuates voltage-dependent Ca^{2+} flow

Concentrations of glutamate (100 μM to 500 μM) that are known to change the membrane potential of NM neurons (Raman and Trussell, 1992; Trussell et al., 1993) do not reliably increase $[Ca^{2+}]_i$ (Zirpel et al., 1994b). Reliable changes in $[Ca^{2+}]_i$ can be produced by 5 mM glutamate. While submillimolar concentrations of glutamate were ineffective in stimulating Ca^{2+} influx, they were effective in altering the *flow* of Ca^{2+} through VOCCs. Several tests were conducted to show that an attenuation of Ca^{2+} influx by glutamate was not mediated by ionotropic glutamate receptors. Ca^{2+} influx was suppressed by quisqualate and *t*-ACPD, agonists of mGluRs. Finally, the attenuation of Ca^{2+} influx was not blocked by CNQX, an antagonist of non-NMDA-type receptor-operated channels (ROCs), or by L-AP3 or L-AP4, which block mGluR actions in other systems (Schoepp and Johnson, 1989; Forsythe and Clements, 1990; Schoepp et al., 1990; Baskys and Malenka, 1991; Tanabe et al., 1991; Trombley and Westbrook, 1992).

Figure 5 plots the Ca^{2+} responses of NM neurons depolarized by 60 mM KCl before and after glutamate superfusion. These responses are representative of the 57 neurons (n = 7) that were tested after superfusion of 200 μM to 500 μM glutamate. Following glutamate exposure, an attenuation in Ca^{2+} influx due to KCl was seen: 72% of the depolarized neurons responded with small increases in $[Ca^{2+}]_i$, averaging 102 ± 32 nM. The remainder showed a larger influx of Ca^{2+}, averaging 310 ± 73 nM. The Ca^{2+} response seen in the other set of neurons developed slowly to a plateau then decreased gradually to basal levels once KCl stimulation was removed. Ca^{2+} transients were never produced by depolarizations administered after glutamate superfusion. A long exposure to glutamate, 3 to 5 min in duration, was required to attenuate the influx of Ca^{2+}. As illustrated in Figure 5*A*, the effect was also long lasting. It was also reversible (not illustrated), returning to prestimulus levels 40 to 50 min after glutamatergic stimulation. Figure 5*B* shows that glutamatergic actions were not dependent upon extracellular Ca^{2+}, indicating that calcium/calmodulin-dependent protein kinases, which have been shown to play a role in mGluR-mediated inhibition of Ca^{2+} influx through L-channels (Lester and Jahr, 1990; Chernevskaya et al., 1991; Nistri and Cherubini, 1991; Zeilhofer et al., 1993), do not play a role in this particular system.

Lastly, Figure 6*A* summarizes the effects that different VOCC antagonists had on the KCl-induced Ca^{2+} response *following* glutamate exposure. A total of 36 neurons (n = 3) were examined. The glutamatergically attenuated Ca^{2+} response was not reduced further by nifedipine: following glutamate exposure, 60 mM KCl increased $[Ca^{2+}]_i$ to 288 ± 88 nM; the $[Ca^{2+}]_i$ rose to 257 ± 91 nM in glutamatergically treated neurons that were simultaneously exposed to 60 mM KCl and 1 μM nifedipine. The difference was not statistically significant. Attenuated Ca^{2+} responses were reduced further by pimozide (see Fig. 6*B*), a diphenylbutylpiperidine that preferentially (albeit nonspecifically) blocks low voltage-activated calcium channels (Enyeart et al., 1990; Enyeart et al., 1993): 25 μM pimozide further reduced the glutamatergically attenuated Ca^{2+} response from roughly 250 to 93 ± 25 nM. This concentration was not statistically different from basal levels. These results indicate that glutamate modulates L-type channels.

QA and t-ACPD also block VOCCs. Seven slices were exposed to glutamate agonists KA (n = 2), AMPA (n = 2), and

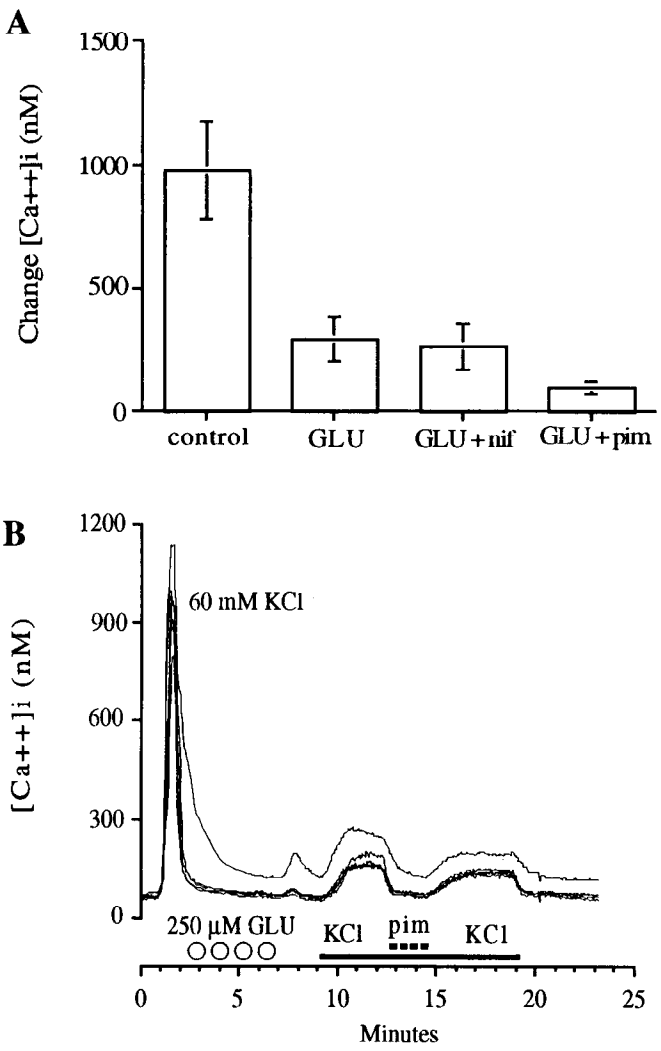

Figure 6. Glutamate modulates L-type channels. *A,* This histogram summarizes the mean elevation in $[Ca^{2+}]_i$ (± SEM) induced by KCl under four separate conditions: *control,* KCl before glutamate superfusion; *GLU,* KCl after a 5 min glutamate superfusion; *GLU + nif,* coapplication of KCl and nifedipine after glutamate superfusion; *GLU + pim,* coapplication of KCl and pimozide after glutamate superfusion. *B,* Shown are Ca^{2+} responses of five neurons to 60 mM KCl before and after superfusion of 250 μM glutamate (indicated by the *white circles*). The first Ca^{2+} transient was produced by a 20 sec-long exposure to KCl, the second transient was produced by a 1 min long exposure to KCl. The *black bar* indicates the period of time 60 mM KCl was continuously superfused. When 25 μM pimozide was coapplied (indicated by *dashed line*) during the KCl stimulus, the $[Ca^{2+}]_i$ was reduced to near basal levels. The $[Ca^{2+}]_i$ elevated after the pimozide was removed.

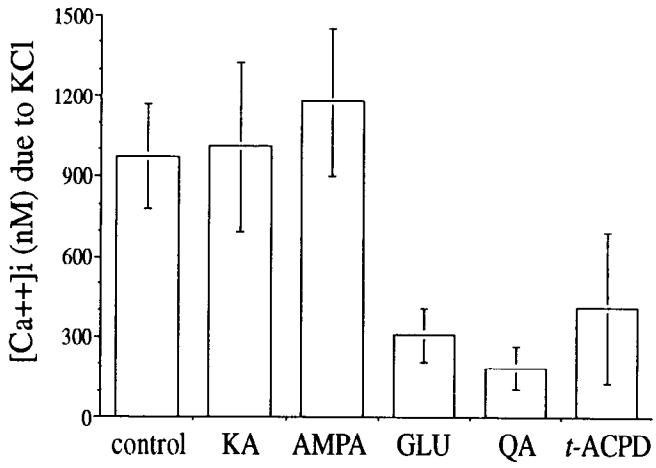

Figure 7. Stimulation of a mGluR is required to attenuate voltage-dependent Ca^{2+} influx. Summarized by this histogram are the mean (± SEM) $[Ca^{2+}]_i$ seen in neurons depolarized by 60 mM KCl alone (*control*), or by KCl immediately after a 3 to 5 min exposure to 25 μM KA or AMPA, to 250 μM glutamate (*GLU*), to 25 μM quisqualate (*QA*), or to 100 μM *t*-ACPD. The magnitude of voltage dependent Ca^{2+} influx was reduced in NM neurons superfused with GLU, QA, or *t*-ACPD. A Student's *t* test revealed that the difference in Ca^{2+} influx following GLU or QA exposure was statistically significant ($p < 0.001$) compared to controls; the voltage-dependent influx of Ca^{2+} observed following *t*-ACPD superfusion, while attenuated compared to controls, did not differ statistically ($p < 0.08$).

QA ($n = 3$), then depolarized with KCl. These results are summarized in Figure 7. Slices were not exposed to NMDA because it is not clear whether NM neurons express functional NMDA-type receptors beyond embryonic day 14 (Nemeth et al., 1983; Raman and Trussell, 1992; Zhou and Parks, 1992). Previous studies have shown that, unlike glutamate, brief exposure to 25 μM KA, 25 μM AMPA, and 25 μM QA causes an increase in $[Ca^{2+}]_i$ (Zirpel et al., 1994b). These concentrations of KA or AMPA did not alter voltage-dependent Ca^{2+} influx. Quisqualate, on the other hand, produced a long-lasting and reversible reduction in Ca^{2+} influx through VOCCs (see Fig. 7). The actions of QA were consistently and reliably produced when QA was coapplied with 50 μM CNQX to block QA-stimulated ionotropic receptor channels.

Six slices were exposed to 100 μM *t*-ACPD then depolarized. Like glutamate, *t*-ACPD (50 μM to 200 μM) did not evoke a Ca^{2+} response in NM neurons (see Zirpel et al., 1994b, for details). Unlike glutamate, *t*-ACPD at these concentrations *reduced* the $[Ca^{2+}]_i$ below basal levels to 42 ± 27 nM. This reduction in basal $[Ca^{2+}]_i$ was not statistically different from normal. The effects of 100 μM *t*-ACPD were examined on 27 NM neurons ($n = 5$). In 66% of these neurons *t*-ACPD significantly decreased the KCl evoked influx of Ca^{2+} (see Fig. 7). The remainder responded to the KCl stimulus with large transients. Higher concentrations of *t*-ACPD were less effective at reducing the KCl-evoked Ca^{2+} transients.

Channel inhibition is not blocked by iGluR antagonists. Concentrations of CNQX that block KA- or AMPA-evoked Ca^{2+} responses (Zirpel et al., 1994b) did not reverse glutamatergic inhibition of Ca^{2+} influx. A total of five slices were incubated in 25 μM CNQX + 250 μM glutamate, then depolarized by KCl. Not one of the 39 neurons examined responded with a Ca^{2+} transient. These results are summarized in Figure 8*A*.

The effects of L-AP3 (Schoepp and Johnson, 1989; Schoepp et al., 1990), L-AP4, and SOP (Nicolletti et al., 1986; Winder et al., 1993), which block the actions of different mGluRs, are summarized in Figure 8*B*. The actions of L-AP3 were examined on a total of 25 neurons ($n = 5$). Not one of these neurons showed a Ca^{2+} transient in response to 60 mM KCl subsequent to coapplication of 200 μM glutamate and 1 mM L-AP3. The actions of L-AP4 were examined in 33 neurons ($n = 6$). Like L-AP3, L-AP4 did not alter the inhibitory actions of glutamate on VOCCs. Finally, the effects of SOP were examined on 27 neurons ($n = 7$). Each neuron responded with a Ca^{2+} transient each time it was stimulated by KCl.

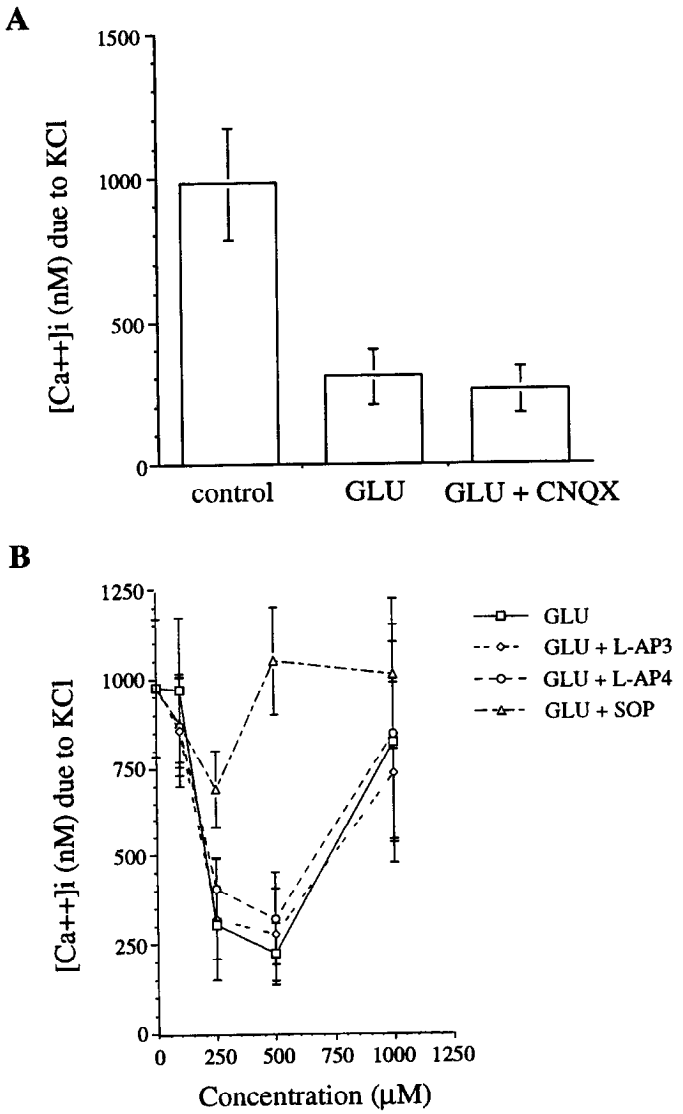

Figure 8. Glutamatergic actions on voltage-dependent Ca^{2+} influx are antagonized by phosphoserine. *A*, The magnitude of Ca^{2+} influx caused by 60 mM KCl following a 3 min exposure to 250 μM glutamate (*GLU*) or to 25 μM CNQX + 250 μM GLU is compared to changes in $[Ca^{2+}]_i$ produced by KCl in untreated slices (*control*). *B*, The magnitude of Ca^{2+} influx caused by 60 mM KCl following a 3 min exposure to 250 μM glutamate (*GLU*) alone, or glutamate + L-AP3, glutamate + L-AP4, or glutamate + SOP, are shown here, presented as dose–response curves. Glutamatergic inhibition of voltage-dependent Ca^{2+} influx is most effective at concentrations less than 500 μM, and is inhibited by serine-*O*-phosphate, not L-AP3 or L-AP4.

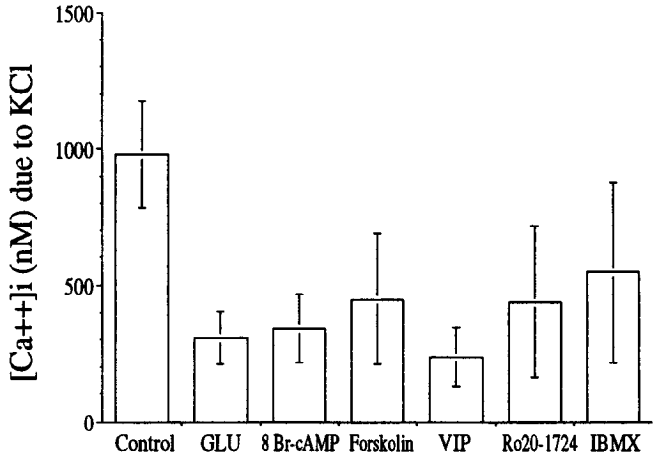

Figure 9. Accumulators of cAMP attenuate voltage-dependent Ca^{2+} influx. This histogram summarizes the mean changes (± SEM) in $[Ca^{2+}]_i$ seen in neurons depolarized by 60 mM KCl alone (*control*), or by KCl immediately after a prolonged exposure to 250 μM glutamate (*GLU*), to 1 mM 8 Br-cAMP, to 50 μM forskolin, to 1 μM VIP, to 250 μM Ro 20-1724, or 100 μM IBMX. The magnitude of voltage-dependent Ca^{2+} influx that was reduced in NM neurons by 8 Br-cAMP and VIP, was similar to that produced by GLU, and were statistically different from control levels ($p < 0.001$). While forskolin attenuated Ca^{2+} influx, the difference was not statistically significant ($p < 0.07$). The phosphodiesterase inhibitors also attenuated Ca^{2+} influx, but not as effectively as GLU, or the other cAMP-accumulators, and their overall effect on Ca^{2+} influx compared to controls was not statistically significant.

Voltage-dependent Ca^{2+} influx is attenuated by cAMP

mGluR activation modulates a variety of intracellular messengers. The most widely recognized mGluR effect is the stimulation of IP3 formation via activation of a phospholipase C (PLC) signal transduction cascade (Schoepp and Conn, 1993, for review). Other mGluRs activate phospholipase D (Boss and Conn, 1992; Holler et al., 1993), inhibit forskolin-stimulated adenylate cyclase activity (Schoepp et al., 1992; Tanabe et al., 1992; Schoepp and Johnson, 1993) or stimulate cAMP accumulation (Goh and Ballyk, 1993; Winder and Conn, 1993; Winder et al., 1993). The experiments described below suggest that an accumulation of cAMP may be involved in glutamatergic modulation of VOCC.

That an increase in $[Ca^{2+}]_i$ was not required to attenuate voltage-dependent Ca^{2+} influx (see Fig. 5*B*) indicated that IP3, a product of the PLC signal transduction cascade, was not involved in glutamatergic modulation of Ca^{2+} channels. To determine whether diacylglycerol (DAG) and its effector, protein kinase C (PKC), played any role in VOCC inhibition, 26 neurons ($n = 4$) were incubated in the PKC-activating phorbol ester PMA (1 μM) for 12 to 15 min then stimulated with 60 mM KCl. Every one of the neurons exposed to the phorbol ester responded to KCl with a large Ca^{2+} transient. These results indicate that the products of PLC signal transduction system were not involved in glutamatergic inhibition of voltage-dependent Ca^{2+} influx. Considering these findings, voltage-dependent changes in $[Ca^{2+}]_i$ were reexamined in NM neurons with pharmacologically elevated $[cAMP]_i$. This was accomplished by incubating slices for 10 to 17 min in the adenylate cyclase activators forskolin (50 μM), or VIP (1 μM), or in the membrane permeable analog of cyclic AMP, 8-Br-cAMP (1 mM). To determine whether nominal concentrations of cAMP could attenuate KCl-stimulated Ca^{2+} influx, a few slices were incubated for 20 min in phosphodiesterase inhibitors, either Ro 20-1724 (250 μM) or IBMX (100 μM). Following a brief rinse in aCSF, neurons were depolarized with 60 mM KCl, and the ensuing change in $[Ca^{2+}]_i$ was observed. The results of these tests are summarized in Figure 9.

Every neuron incubated in forskolin (53; $n = 7$), VIP (24; $n = 3$), and 8-Br-cAMP (30; $n = 3$) showed basal $[Ca^{2+}]_i$ that was lower (but not statistically different) than normal (treated = 55 ± 21 nM vs. control = 78 ± 25 nM). Neurons incubated in Ro 20-1724 (18; $n = 3$) and IBMX (21; $n = 4$) showed normal basal $[Ca^{2+}]_i$ (82 ± 21 nM). The mean change in $[Ca^{2+}]_i$ in KCl-stimulated neurons incubated in each of the cAMP accumulators

was smaller than that seen in untreated neurons; a nonparametric analysis of variance revealed that the difference between normal and accumulator-treated neurons was statistically different. An a priori comparison matching the effects of the PDE inhibitors against the remaining cAMP accumulators showed that the significant F value produced by the ANOVA was not due to the effects of the PDE inhibitors. Thus, a significant increase in $[cAMP]_i$ (which could not be completely produced by reducing PDE activity) was required to attenuate voltage-dependent changes in $[Ca^{2+}]_i$. Post hoc comparisons testing the individual effects of forskolin, VIP, and 8-Br-cAMP revealed that the attenuation of Ca^{2+} influx caused by forskolin was not statistically different from normal, while the changes due to VIP or 8-Br-cAMP were. It is notable that 58% of the forskolin-treated neurons did not show a Ca^{2+} transient when depolarized; the remainder responded with a large transient that was eliminated following a brief (1 min) superfusion of 100 μM glutamate + 10 μM forskolin. The effect of glutamate and forskolin on this second set of NM neurons was synergistic, as neither 100 μM glutamate by itself (see Fig. 8) or 10 μM forskolin by itself, could block the KCl-stimulated changes in $[Ca^{2+}]_i$.

Discussion

Glutamate stimulates two different receptor types (Sugiyama et al., 1989): ionotropic receptors, linked to ion channels, and metabotropic receptors, which activate signal transduction cascades. In this study, the ratiometric Ca^{2+} indicator dye Fura-2 was used to show that concentrations of glutamate that produce a depolarization in NM neurons (Raman and Trussell, 1992) attenuates Ca^{2+} influx through voltage-sensitive channels, via the activation of a mGluR. The main results show that (1) NM neurons possess high voltage-activated Ca^{2+} channels; (2) the inhibition of Ca^{2+} influx caused by nifedipine and glutamate are equivalent; (3) glutamatergic attenuation of Ca^{2+} influx is long lasting, and duplicated by quisqualate and t-ACPD, but not KA or AMPA, and antagonized by serine-O-phosphate; (4) Ca^{2+} influx is also inhibited by increasing $[cAMP]_i$. It is important to add that a significant inhibition of Ca^{2+} influx cannot be achieved by inhibiting PDE activity. Thus, one possible explanation for the glutamatergic inhibition of voltage-dependent Ca^{2+} influx is that mGluR stimulation positively modulates adenylate cyclase activity, rather than negatively regulating the actions of PDEs.

This discussion addresses three topics. First, which mGluR produces the actions described in NM? The signal transduction pathways stimulated by seven cloned mGluRs, designated mGluR1 through mGluR7, have been identified (Okamoto et al., 1994; and for review, see Schoepp and Conn, 1993). Phospholipase C is activated following stimulation of mGluR1 and mGluR5 (Sugiyama et al., 1989; Masu et al., 1991; Abe et al., 1992; Aramori and Nakanishi, 1992). The remainder activate an inhibitory cAMP cascade (Nakajima et al., 1993; Tanabe et al., 1992, 1993; Okamoto et al., 1994). That accumulators of cAMP attenuate voltage-dependent Ca^{2+} influx indicates that the NM mGluR may modulate VOCCs by increasing the activity of the adenylate cyclase signal transduction system. Secondly, which Ca^{2+} channel is modulated? Studies examining the actions of mGluRs on VOCCs conclude that N-channels are modulated. In NM neurons, mGluR stimulation appears to modulate L-channels. Finally, the functional significance of mGluR activation is discussed. Inhibition of VOCCs by mGluRs is believed to attenuate neurotransmission. In NM neurons, postsynaptic channels appear to be modulated, and the second messengers involved in this modulation may be a critical link in the pathway for transneuronal degeneration due to activity deprivation.

mGluR stimulation inhibits calcium channels in NM neurons

Of the seven different mGluRs that have been cloned, only mGluR1 stimulates cAMP accumulation (Aramori and Nakanishi, 1992). Although mGluR1 increases $[cAMP]_i$, has the same potency rank order of agonists that inhibit Ca^{2+} influx in NM neurons, and is invulnerable to L-AP3 or L-AP4, two facts indicate that a different mGluR modulates Ca^{2+} channels in NM. First, mGluR1 hydrolyses phosphatidylinositol-4,5-bis-phosphate, leading to an increase in PKC activity and $[Ca^{2+}]_i$ (liberated from internal stores). Neither PKC nor an increase in $[Ca^{2+}]_i$ were required to inhibit Ca^{2+} influx. Secondly, the concentration of t-ACPD required to elevate $[cAMP]_i$ in mGluR1-expressing oocytes is 10 times greater than that required to stimulate IP3 formation (Aramori and Nakanishi, 1992). While products of PI hydrolysis can be generated in explants of NM exposed to less than 100 μM t-ACPD (Zirpel et al., 1994a), 1 mM t-ACPD is required to mobilize sequestered Ca^{2+} in NM neurons (Zirpel et al., 1994b). This concentration was not used in this study because t-ACPD concentrations exceeding 200 μM did not attenuate voltage-dependent Ca^{2+} influx effectively. Glutamatergic actions also became less effective at higher concentrations (see Fig. 8B). These results suggest that two or more mGluRs may be expressed in NM neurons. One stimulates IP3 formation; the second results in an increase in cAMP. In NM, the cAMP accumulating mGluR also acts independent of Ca^{2+}, and is antagonized by the endogenous metabolite, SOP.

An SOP-sensitive cAMP-accumulating mGluR has also been described in the hippocampus (Winder and Conn, 1993; Winder et al., 1993). It is noteworthy that lower concentrations of glutamate and t-ACPD are also more effective at potentiating cAMP responses in hippocampal neurons, while significantly higher concentrations of glutamate and t-ACPD are required to stimulate phosphoinositide hydrolysis (Winder et al., 1993). However, unlike the NM mGluR, in the hippocampus, increasing concentrations of glutamate and t-ACPD increases cAMP accumulation in a dose-dependent fashion. Higher concentrations of glutamate and t-ACPD were less effective at attenuating Ca^{2+} influx in NM, and presumably stimulating cAMP activity. This U-shaped dose–response relationship suggests that the NM mGluR is only active during periods when submillimolar concentrations of glutamate are in the synaptic cleft. Thus, one possible role for this mGluR is that it acts in conjunction with glutamate reuptake, and receptor desensitization systems, to protect the neurons from the effects of constant, low-level exposure to glutamate.

mGluRs block L-channels in NM neurons

Two types of voltage-operated calcium channel exist: T channels, activated by small depolarizations, and a trio of channels, DHP-sensitive L-channels, ω-CgTX-sensitive N-channels, and FTX-sensitive P-channels, activated by larger depolarizations. The following results indicate that glutamate impedes Ca^{2+} influx through L-channels in NM neurons. First, Ca^{2+} influx observed in depolarized neurons exposed to nifedipine and glutamate was identical; in either case, large Ca^{2+} transients were eliminated. Second, the voltage-dependent change in $[Ca^{2+}]_i$ seen following glutamate treatment could not be further reduced by nifidipine; it was reduced to nearly basal levels by pimozide,

a nonspecific antagonist of low voltage-activated channels. Finally, the N-channel blocker ω-CgTX did not suppress KCl-stimulated Ca^{2+} influx. A test for the presence of P-channels, which may be involved in transmitter release from the auditory nerve (Jackson and Parks, 1989), was not undertaken.

The subject of neurotransmitters altering Ca^{2+} channel function has been extensively studied. GABA (Scott and Dolphin, 1990), acetylcholine (Bernheim et al., 1991), norepinephrine (Lipscombe et al., 1989), and serotonin (Braha et al., 1993) all inhibit VOCCs. Glutamate has recently been added to this list (Lester and Jahr, 1990; Sayer et al., 1992; Swartz and Bean, 1992; Haws et al., 1993; Sahara and Westbrook, 1993). There is widespread agreement that glutamatergic actions are regulated by a mGluR because they (1) can be duplicated by the specific mGluR agonist *t*-ACPD, (2) cannot be duplicated by KA, AMPA, or NMDA, and (3) cannot be blocked by iGluR antagonists. Identical criteria have been used to characterize glutamatergic actions on L-channels in NM neurons.

While PKC has been shown to inhibit Ca^{2+} currents/influx (Anwyl, 1991; Doerner and Alger, 1992; Haymes et al., 1992; Swartz, 1993; Swartz et al., 1993), PKC has not been implicated in the mGluR-mediated VOCC inhibition that has been described previously in the mammalian models (Lester and Jahr, 1990; Sayer et al., 1992; Swartz and Bean, 1992; Sahara and Westbrook, 1993). In NM neurons, Ca^{2+}-channel inhibition could be mediated by cAMP. An enhancement of whole cell Ca^{2+} current responses, or a potentiation of Ca^{2+} influx through ionotropic receptors is usually associated with cAMP or its effector, PKA (Artalejo et al., 1990; Greengard et al., 1991; Wang et al., 1991; Bleakman et al., 1992; Keller et al., 1992). PKA-mediated Ca^{2+}-channel inhibition does not appear to be unique to NM neurons, however; dopaminergic stimulation of cAMP accumulation inhibits Ca^{2+} channels in other systems (e.g., Liu and Lasater, 1994).

Previous studies of mGluR-mediated L-channel inhibition may have overlooked the contributions of cAMP. This is understandable because mGluR stimulation is normally associated with the liberation of sequestered Ca^{2+}. Additionally, five of the seven cloned metabotropic receptors inhibit cAMP accumulation. Excitation of the cAMP cascade by mGluRs has only recently been reported (Goh and Ballyk, 1993; Winder and Conn, 1993; Winder et al., 1993; Musgrave et al., 1994). There is also some evidence indicating that the activation of the adenylate cyclase system by metabotropic receptors is developmentally regulated (Casabona et al., 1992).

Functional significance

In addition to inhibiting N- and L-channels, mGluRs also suppress excitatory postsynaptic potentials (Desai and Conn, 1991; Lovinger, 1991; Glaum et al., 1992). The combination of these actions suggests that mGluRs act presynaptically to block neurotransmission. Our results suggest that mGluRs act postsynaptically in NM. If the Ca^{2+} influx seen in KCl-depolarized NM neurons was due to the release of neurotransmitter from auditory nerve terminals, then this influx should have been suppressed or eliminated by ω-CgTX. There is widespread acceptance (although it has not been directly examined in NM) that presynaptically located ω-CgTX–sensitive N-type calcium channels regulate neurotransmission (see Bean, 1989, for review). ω--CgTX did not affect NM neurons' Ca^{2+} responses to KCl. Furthermore, NM neurons exhibit a very high rate of spontaneous activity (Rubel and Parks, 1975; Warchol and Dallos, 1990), which is totally eliminated by removing or silencing auditory nerve input (Born et al., 1991; Lippe, 1994). Finally, glutamate has been implicated as a trophic factor for NM neurons (Hyson and Rubel, 1989). Thus, a mGluR that attenuates neurotransmission would be undesirable in NM.

In order to understand the importance of a postsynaptically located L-channel modulating mGluR, it is useful to note that NM neurons are responsible for faithfully and precisely transmitting the microsecond differences that separate the arrival of sounds. This task has been simplified by specialized auditory nerve terminations (Carr and Boudreau, 1991), called endbulbs of Held, that cover nearly 60% of the NM neuronal surface (Hackett et al., 1981; Parks, 1981; and see Rubel and Parks, 1988 for review). As a consequence of calyciferous innervation, NM neurons are capable of phase locking to frequencies between 2000 and 9000 Hz (Sullivan and Konishi, 1984; Warchol and Dallos, 1990). An additional consequence of calyciferous innervation is that even in the absence of stimulation, NM neurons remain active: spontaneous rates exceed 100 Hz (Sullivan and Konishi, 1984; Warchol and Dallos, 1990). That auditory nerve terminals release glutamate (Nemeth et al., 1983; Raman and Trussell, 1992; Zhou and Parks, 1992), which can only be removed by diffusion from the cleft [which may not occur rapidly (Trussell et al., 1993)] or by reuptake into the presynaptic terminal, places NM neurons in a potentially excitotoxic environment. In fact, it is possible that a glutamate "sink" might exist in the synaptic cleft linking NM with the auditory nerve calyx because the rate of activity is so high, and the area of the auditory nerve synapse is so great (see Trussell et al., 1993). The notion of a glutamate sink is worth some consideration when the rates of driven activity are linked with the possibility that millimolar concentrations of glutamate are expelled from individual vesicles (Clements et al., 1992). Even under the best reuptake conditions, it might be difficult to completely purge the cleft of the glutamate. Thus, the cAMP-accumulating mGluR may be activated to protect NM neurons from this ever-present concentration of glutamate that has the potential of depolarizing the neuron and allowing Ca^{2+} to accumulate to lethal levels.

Continuous activation of this mGluR appears to be important for NM neurons. If glutamatergic stimulation is eliminated by cochlear removal or blocking auditory nerve action potentials with TTX, NM neurons immediately initiate a cascade of events culminating in the death of 30% of the neurons (Rubel et al., 1990, for review). In an *in vitro* slice preparation developed to study signals that regulate activity-controlled neuronal degeneration (Hyson and Rubel, 1989), we found that NM neurons remained viable as long as they were stimulated orthodromically. Antidromically stimulated NM neurons began to deteriorate. In light of the present results, it would be important to determine if antidromically stimulated NM neurons remained viable in the presence of a Ca^{2+} channel blocker, a mGluR agonist, or an accumulator of cAMP. Thus, it appears that the essential message glutamate provides to NM neurons is conveyed through its metabotropic regulation of cAMP.

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